Bacterial Lipid Domains and Their Role in Cell Processes

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Abstract
Bacterial plasma membranes, mainly composed of phospholipids and proteins, separate the interior of a cell from their environment and maintain cell homeostasis. Early notions of membrane organization gave rise to a model in which a homogeneous lipid bilayer permits free protein diffusion within the membrane. However, proteins and phospholipids are distributed unevenly in bacterial membranes, and specific membrane localization is often crucial for protein activity or function. Bacterial membrane domains with different lipid compositions and with differential physical properties in comparison with the surrounding membrane have now been described. These membrane domains appear to influence...
1 Introduction

Cellular plasma membranes serve to delimit the interior of a cell from the exterior environment. For decades these plasma membranes were thought to be a two-dimensional homogeneous and dynamic assortment of phospholipids (PL) and proteins, as proposed by the Singer and Nicolson fluid mosaic model (Singer and Nicolson 1972). According to this model the lipid bilayer functions as a neutral two-dimensional solvent, having no influence on membrane protein function. During the late 1980s and 1990s, however, the fluid mosaic model was challenged by numerous studies that provided evidence for specialized membrane structures, with differential composition and fluidity from the rest of the membrane, within the lipid bilayer of eukaryotic membranes (Simons and Van Meer 1988; Brown and Rose 1992; Simons and Ikonen 1997). These membrane platforms, coined lipid rafts, were shown to have the ability to spatially organize proteins, by including or excluding specific proteins to variable extents, thereby promoting kinetically favorable interactions (Allen et al. 2007) and regulating a variety of cellular functions. The fact that the composition of these eukaryotic membrane platforms contained invariably glycosphingolipid and cholesterol that are absent in almost all bacterial membranes, diverted attention on possible bacterial membrane domains. Nevertheless the development of new lipid staining techniques and imaging technologies, during the last decade, allowed the observation of specific membrane regions with different characteristics. These observations were initially interpreted as heterogeneous distribution of different lipid species in bacterial membranes, thus giving rise to the notion of bacterial membrane domains (Fishov and Woldringh 1999; Mileykovskaya and Dowhan 2000; Barák et al. 2008). Moreover, it was observed that bacterial membranes have heterogeneous protein distribution, as various proteins were found to localize to specific sites on the plasma membrane. Finally, several reports in the few last years, provided evidence that bacterial membrane domains are crucial for many physiological processes, such as cell division, cell wall biosynthesis, signal transduction, protein secretion, among others (Barák and Muchová 2013). Here, the different types of bacterial membrane domains will be described, and their importance for various cellular processes will be discussed.

2 Membrane Composition

The most abundant lipid components of biological membranes are phospholipids, which are amphipathic molecules that tend to spontaneously form a bilayer in an aqueous environment. The major lipid class defining this bilayer in practically all localization, diffusion, and function of membrane proteins, and thereby seem to be involved in many cellular processes. Here, we describe the different types of bacterial membrane domains and discuss their involvement in various bacterial cellular processes.
membranes is the glycerophospholipid. This lipid is based on an esterified glycerol moiety with two acyl-chains (fatty acids) and with a phosphate group. The phosphate group is bound to a specific alcohol head group, resulting in the different types of glycerophospholipids, i.e., phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidic acid (PA), etc. (Fig. 1) (Sohlenkamp and Geiger 2016). In addition to glycerophospholipids, other lipids, such as polyisoprenoids and hopanoids in bacteria as well as sterols and the ceramide-based sphingolipids in eukaryotic membranes, but exceptionally also in some bacterial membranes, are usually found to form part of biological membranes (Fig. 1) (van Meer et al. 2008; Sohlenkamp and Geiger 2016).

Lipids account for almost half the mass of a membrane, whereas proteins account for the other half. Many of these proteins have one or more α-helices inserted into the lipid bilayer and can only be separated from it by detergent treatments. These integral proteins include transporters, electron carriers, signaling and structural proteins, among others.

Curiously, many proteins, known as peripheral proteins, that have been shown to co-localize with PG or CL are apparently soluble proteins, lacking any obvious transmembrane region. A common characteristic of such proteins is that all of them have at least one amphipathic α-helix, which can be located either at the C- or N-terminus, and mediates interactions with the lipid bilayer (Orgel 2006). These amphipathic α-helices, known also as membrane-targeting sequences (MTS), are necessary and sufficient to achieve membrane association of proteins. In MTSs, one face of the helix is highly hydrophobic whereas the other one is strongly polar, usually containing positive charged residues. This characteristic explains the anionic-PL affinity to MTSs, depending on hydrophobic interactions between one face of the helix and the acyl-chains of PL, and on electrostatic interactions due to the net positive charge of the polar face of the helix and the negative charge of the head group of the PG or CL domain. MTSs are found in a variety of proteins such as MinD (Szeto et al. 2002), the actin homologues MreB (Salje et al. 2011) and FtsA (Pichoff and Lutkenhaus 2005), the CL synthase ClsA (Kusaka et al. 2016), the exoribonuclease RNaseII (Lu and Taghbalout 2013), among others. The physiological role of some of them and their possible involvement in the coordination of lipid domain formation in Escherichia coli and Bacillus subtilis will be discussed below.

3 Bacterial Membrane Heterogeneities

3.1 Bacterial Lipid Bilayers and Transversal Asymmetry

Specific staining techniques and microscopic observation constituted the first approaches to study bacterial envelopes. In 1884, the Danish bacteriologist Hans Christian Gram developed a staining technique that is used until the present day. Depending on their capability to retain the Gram stain (the crystal violet dye), most bacteria fall into one of two main groups: Gram-negative or Gram-positive bacteria. This differential feature relies on essential structural differences in the cell envelope.
Phospholipids

Sterol and putative functional analogues in bacteria

Fig. 1 (continued)
Gram-negative bacteria, such as *E. coli*, are surrounded by a lipopolysaccharide containing membrane (outer membrane, OM), a thin peptidoglycan cell wall, and a cytoplasmic phospholipid bilayer or inner membrane (IM). In contrast, Gram-positive bacteria have a single membrane that is surrounded by a thick peptidoglycan wall. The OM of *E. coli* and many other Gram-negative bacteria is a highly asymmetric bilayer, with phospholipids predominantly on the inner leaflet and lipopolysaccharides (LPS) on the outer one. This composition confers to OM and the proteins embedded therein, natural resistance to solubilization by detergents, such as bile salts, a property that enables bacteria to survive in the mammalian gut. Asymmetry has also been observed in bacterial cytoplasmic membranes, with PE being preferentially situated in the inner leaflet and PG in the outer face of the lipid bilayer (Rothman and Kennedy 1977). In addition, it was proposed that CL is predominantly distributed on the inner leaflet in bacterial cytoplasmic membranes (McAuley et al. 1999; Huang and Ramamurthi 2010). Taken into account that CL accumulates in polar regions of rod-shaped bacteria (see below), this asymmetry in CL distribution, with preference to the concave cytoplasmic leaflet of the bilayer, is consistent with the predicted conical shape of a CL molecule, composed by two phosphatidyl moieties, four acyl chains and a relatively small polar head group.

Interestingly, asymmetry in the distribution of phospholipids in both leaflets of eukaryotic membranes has been shown to be actively maintained by the activity of lipid translocases and to promote the formation of ordered micro-domains (lipid rafts) (Cheng et al. 2009). However, no data exists from bacterial cytoplasmic membranes relating transversal asymmetry to membrane micro-domain assembly.

### 3.2 Uneven Distribution of Anionic Phospholipids

The *E. coli* cytoplasmic membrane is composed mainly of three glycerophospholipid species, the zwitterionic PE, representing the 75% of the total membrane phospholipids, and the anionic PG and CL, accounting for 20% and 5%, respectively (Raetz and Dowhan 1990). However, the membrane composition not only differs strongly from one taxonomic group to another and between genera from the same group, but also in the same strain depending on the physiological conditions. Consequently, the question of whether the different phospholipid species are homogeneously distributed across the membrane or whether determined lipids are enriched in specific regions of the membrane was raised. Indeed, by using dyes with different lipid specificities, PG and CL enriched domains were observed to be located in specific membrane regions. One of the first agents used to study the organization of bacterial membranes was FM 4–64, which is a positively charged molecule and it was therefore believed to bind anionic lipids, such as PG and CL. Fishov and Woldringh

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**Fig. 1** Chemical structures of the major bacterial phospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and representative structures of sphingolipid (Sph), cholesterol, hopanoid and carotenoid are presented.
(1999) observed a pattern with dark bands between bright regions in FM 4–64 stained membranes of living E. coli cells. It was therefore assumed that the dark bands represented PE enriched regions, whereas the brightest areas, which were close to the cell poles, represented PG and CL enriched regions (Fishov and Woldringh 1999). Later on, by the use of the membrane dye 10-N-Nonyl acridine orange (NAO), which was suggested to have a selective fluorescent emission in the presence of CL, membrane regions enriched in CL were observed at cell poles and septal regions of E. coli (Mileykovskaya and Dowhan 2000, 2009), Pseudomonas putida (Bernal et al. 2007), B. subtilis (Kawai et al. 2004) and Mycobacterium tuberculosis (Maloney et al. 2011). The preferential localization of CL at cell poles in E. coli was confirmed by later studies and with different techniques. On the one hand, lipid analysis of minicells, in contrast to vegetative cells, showed that they are enriched in CL (Koppelman et al. 2001). Minicells are spherical and anucleated membrane vesicles that are produced by inactivation of any of the minC, minD or minE genes. These mutations cause a shift in the division site from mid-cell to the vicinity of the cell poles, and therefore the membrane of polar regions of normal cells are well represented in minicell membranes. On the other hand, in vitro experiments demonstrated that changes in membrane curvature cause a change in CL localization, displaying a significant preference to localize at negative curvatures encountered in the inner leaflet of the IM of the E. coli cell poles (Renner and Weibel 2011). Nevertheless, bacteria devoid of CL are essentially healthy cells under lab growth conditions, and their content of PG appears to increase in a compensatory manner. Curiously, in these cells, NAO-stained enriched regions were still observed, casting doubt on the use of NAO as a specific CL-staining agent. In fact, NAO was found to bind promiscuously to other anionic PLs, such as PG and PA, producing nearly the same spectroscopic changes and rendering it impossible to differentiate between these lipid species (Oliver et al. 2014). In addition, E. coli cells devoid of both CL and PG are still viable, and compensate this deficiency by accumulating between 5% and 10% of other anionic PLs such as phosphatidic acid (PA) and N-acylphosphatidylethanolamine (N-acyl-PE), which are also located principally at cell poles and septal regions (Mileykovskaya et al. 2009). Thus, it seems most likely that bacterial cells need anionic PLs to carry out essential physiological processes, as anionic PL domains may play a vital role providing the required environment for the function of many proteins.

3.3 Physiological Roles of Anionic PL Domains

Several studies, in the last two decades, provided evidence for the involvement of CL or PG in several cellular functions. Many of these evidences came from studies in which CL or PG were found to co-purify or were found to be physically associated with a variety of membrane proteins. Moreover, anionic PLs were shown to modulate enzymatic activities or to be required for the reconstitution of active proteins in vitro (Arias-Cartín et al. 2012). For instance, the Mg2+ transporter MgtA of E. coli co-localizes with CL domains at the cell poles and requires CL or PG to be
reconstituted as an active ATPase in vitro (Subramani et al. 2016). Another example is provided by the association of anionic PLs with the protein translocation machinery, consisting of the SecYEG complex and the ATPase SecA. CL, and to a lesser degree PG, were shown to associate with the SecYEG translocon, thereby stabilizing its active dimeric form, and to stimulate the in vitro ATPase activity of SecA (Gold et al. 2010). Curiously, in B. subtilis the PG pool, instead of CL, was found to improve SecA activity and to control its localization, thereby affecting the whole translocon behavior (Campo et al. 2004). A third example for a physiological role is the association of CL with the osmotic stress response in E. coli. Under increasing osmotic pressure bacteria respond by adjusting the distribution of solutes (osmolytes) across the membrane. In E. coli, this is achieved by ProP, which acts as an osmosensory transporter that allows the cytosolic accumulation of osmolytes. Under such osmotic stress conditions it was noted that the expression of the cls genes, encoding for CL synthases, is activated, leading to a significant increase in CL content. Curiously, ProP was found to localize in the cell poles, a CL-rich membrane region, on a CL-dependent manner, as no polar localization of ProP was observed in clsA mutant cells (Romantsov et al. 2007). However, the activity of ProP appeared to not be affected. This was explained by the fact that inactivation of CL synthesis is compensated by an increase in the amount of PG, another anionic PL, as ProP activity was completely lost in a CL- and PG-deficient strain. Therefore, it was concluded that an anionic PL enriched membrane environment, rather than localization at the cell poles, is required for proper function of ProP (Romantsov et al. 2007, 2009). Thus, anionic PL domains appear to serve as an anchor site for several proteins, leading to their localization to specific sites in the cell, and they seem to be required for their activity.

### 3.3.1 PG Domains and Cell Division: Lipid Spirals in B. subtilis

A last example worth describing in more detail is the process of bacterial cell division, in which MTS containing proteins and anionic lipid domains have been shown to be essential. Cytokinesis is finely coordinated with replication and segregation of the chromosome, ensuring that each daughter cell can receive a copy of the chromosome after division. The earliest step of bacterial cytokinesis is the formation of the membrane-tethered Z-ring at the division site, by the polymerization of the tubulin homologue protein FtsZ, a cytosolic protein. In B. subtilis, membrane tethering of the FtsZ filament depends on FtsA, a MTS containing peripheral protein. Subsequently, the Z-ring acts as a scaffold that recruits more than 10 essential proteins to produce a mature division-machinery that constricts the cell and completes cytokinesis (Aj De Boer 2010).

The nucleoid occlusion (NOC) system and Min system provide the main mechanisms that control the above process. On one hand the Noe protein associates to the membrane by a N-terminal MTS, and at the same time binds to specific sites on the DNA, enabling membrane-chromosome association and physically impeding Z-ring formation in the vicinity of the nucleoid (Adams et al. 2015). On the other hand, the Min system, composed by MinC and MinD, inhibits cell division at the poles while allowing it to occur at mid-cell. MinD, an ATPase that is associated to the inner face
of the membrane by a C-terminal MTS (Szeto et al. 2002), forms extended filaments on the membrane surface. These filaments recruit MinC from the cytosol and activate it as a cell division inhibitor. MinC, which is the effector of the Min system, directly interacts with FtsZ, thus impeding functional associations with FtsA and with itself, thereby impeding Z-ring formation (Djakovic et al. 2008; Shen and Lutkenhaus 2009). A question that comes to mind is how does this system select the lateral positioning of the Z-ring, restricted to be at mid-cell. The DivIVA protein gives the answer to this question. DivIVA associates to the inner face of the plasma membrane, by an N-terminal amphiphatic α-helix, which possesses an intrinsic affinity to negative curvatures, and therefore it accumulates at the cell poles (Lenarcic et al. 2009). Consequently, MinCD recruitment by DivIVA generates a concentration gradient of the first, ensuing a minimum concentration at the cell center where Z-ring formation can take place. It is noteworthy that in *E. coli*, which lacks DivIVA, a dynamic mechanism in which MinE-stimulated oscillatory movements of MinCD produce a concentration gradient of the MinCD complex between cell poles, thereby confining Z-ring formation at mid-cell (Meinhardt and de Boer 2001), [see more references in (Lutkenhaus 2007; Vats et al. 2009; Aj De Boer 2010)].

The MTS-containing protein MinD of *B. subtilis*, which is distributed on the inner face of the membrane, was observed to form spiral-like structures (Barák et al. 2008). Interestingly, by using the positive charged lipid dye FM 4–64 and confocal microscopy, Barak and colleagues described the same spiral-like pattern in *B. subtilis* cells, presumably composed of PG, since NAO-stained cells showed that CL were preferentially distributed in the septal regions and at the poles of cells. Moreover, the authors observed that the MinD spirals co-localized with the lipid helices on the membrane surface. Because depletion of anionic lipids produced a MinD delocalization it was proposed that PG-enriched membrane domains, determine the localization of MinD, and therefore are essential for the functioning of the Min system (Fig. 2) (Barák et al. 2008). Thus, PG domains appear to control the process of cell division by determining the specific localization of the cell division-machinery.

It is worth mentioning that spiral-like distribution on the plasma membrane is not exclusive for MinD, as other MTS-containing proteins such as MreB and SecA exhibit spiral-like patterns (Jones et al. 2001; Campo et al. 2004), highlighting the importance of MTS motifs in the localization of proteins in PG domains.

Considering the above observations, a model providing a physiological link between the heterogeneous distributions of lipid species on the cytoplasmic membrane and essential cellular processes was put forward. In this model, CL-enriched domains seemed to be needed by a variety of proteins to be active at the cell poles and PG-enriched helical domains appeared to localize other proteins that partake in different processes.
Nevertheless, the above model may not apply for Gram-negative bacteria. This is because PG-enriched helical domains could not be observed in *E. coli* cells, in which bands or dots around the membrane were observed after staining with the positive charged lipid dye FM 4–64. This was despite that MinD showed a spiral-like localization (Shih et al. 2003).

Moreover, different MTS-containing proteins were shown to have differential localization patterns. For instance, the CL synthase A, ClsA, of *E. coli* contain a C-terminal MTS but has been shown to be preferentially at cell poles and septum, presumably associated with CL-enriched domains (Kusaka et al. 2016). In another case, the actin-like protein MreB of *E. coli* was shown to be organized forming long helical structures that extend along the length of the cell, similarly to MinD, but curiously these structures did not colocalize, suggesting that both MTS-containing proteins are not part of the same lipid domain (Shih et al. 2005). Hence, in addition to a PG and CL enriched environments other factors may contribute to the proper localization of membrane-associated proteins.
Until recently, membrane domains in bacteria have been thought to be limited to discrete regions enriched in PLs with a specific hydrophilic head. However, recent findings have shifted the focus in the analysis of membrane heterogeneity in bacterial cytoplasmic membranes. That is, physiologically relevant membrane regions with increased fluidity (RIFs) with respect to adjacent areas, and dynamic membrane assemblies with lower fluidity, similar to eukaryotic lipid rafts, were found to exist in bacterial membranes (Fig. 3).

**Fig. 3** Scheme of the organization of bacterial membrane regions with increased fluidity (RIF) and lipid raft-like domains. (Left) MreB-induced RIF affects both the localization and the diffusion of certain membrane proteins. The isoprenoid complex molecule Lipid II, which is a cell wall precursor carrier, has been proposed to be involved in the increase of membrane fluidity. (Right) Lipid raft like domains serve as organizing centers that compartmentalize cellular processes, such as signaling and protein translocation and diffuse laterally as a whole in the lipid bilayer. It has been proposed that hopanoids, carotenoids or other unknown lipid species are necessary for the decreased fluidity of these membrane domains, and that the Stomatin, Prohibitin, Flotillin and HflK/C (SPFH)-containing proteins help to nucleate proteins/protein complexes into them.

### 4 The New Paradigm: Proteins Organize Membrane Domains with Different Fluidity in Bacteria

Until recently, membrane domains in bacteria have been thought to be limited to discrete regions enriched in PLs with a specific hydrophilic head. However, recent findings have shifted the focus in the analysis of membrane heterogeneity in bacterial cytoplasmic membranes. That is, physiologically relevant membrane regions with increased fluidity (RIFs) with respect to adjacent areas, and dynamic membrane assemblies with lower fluidity, similar to eukaryotic lipid rafts, were found to exist in bacterial membranes (Fig. 3).

### 4.1 Membrane Domains with Increased Fluidity

The discovery of membrane regions with increased fluidity came from the study of the bacterial MreB cytoskeleton. MreB, a peripheral protein, is a bacterial actin homologue that is widely distributed in rod-shaped bacteria and has been shown to be involved in cell shape maintenance. Like other MTS-containing proteins, MreB associates reversibly with the inner face of the plasma membrane by hydrophobic
and electrostatic interactions. Subsequently, it interacts with the integral membrane proteins MreC and MreD, and recruits multiple peptidoglycan cell-wall biosynthetic proteins. Intriguingly MreB was observed to form spiral-like filaments in *B. subtilis* (Jones et al. 2001), and since then various models have been put forward for its function and localization [(Errington 2015) and refs therein]. In an actual model, MreB is believed to form extended filaments that move around the membrane in form of coils. MreB filament motion is driven by the peptidoglycan cell wall biosynthetic machinery, and in a direction dictated by the MreB filament (Garner et al. 2011; van Teeffelen et al. 2011). Therefore, the MreB cytoskeleton differs from the cortical actin cytoskeleton of eukaryotic cells. The latter one determines the cell shape by the organization of a network of actin filaments attached to the inner face of the plasma membrane, whereas MreB determines bacterial cell shape by enabling and coordinating cell wall formation.

Recently, Strahl and colleagues observed that delocalization of MreB in *B. subtilis* produces changes in the Nile Red and FM 4–64 staining patterns. Nile red is an uncharged lipid dye that appears to stain uniformly the cell membranes, whereas FM 4–64 was observed to have a helical distribution that was interpreted as PG enriched domains. However, after delocalization of MreB by membrane depolarization, staining with both dyes resulted in fluorescent foci in diverse regions of the cell membrane. These foci were independent of the CL, PG or PE content but were dependent of the presence of MreB (and its paralogs in *B. subtilis*, Mbl and MreBH), with which they were co-localized. Because the same punctuated staining pattern was observed with both dyes, the authors discarded the possibility that the bright foci represent domains enriched in lipids with specific head groups. Instead, by using dyes with high specificity to fluid membrane areas such as Laurdan and DiI-C12, it was noted that the MreB associated foci represented membrane regions with increased fluidity (RIFs). Importantly, using these dyes, RIFs were observed to colocalize with MreB even without membrane depolarization of *B. subtilis* cells (Strahl et al. 2014). Thus, MreB appears to be able to organize membrane domains by increasing local membrane fluidity. This can be achieved either by reorganizing the distribution of fatty acids with more insaturations or with branched chains, or by the association with specialized lipid species. In this respect, the peptidoglycan biosynthesis precursor lipid II, which is a complex isoprenoid lipid linked to a sugar moiety and a pentapeptide, was proposed to be involved in the increase of membrane fluidity associated to MreB filaments (Scheffers et al. 2015; Strahl and Errington 2017). More recently, the role of MreB in membrane organization was also observed in *E. coli* cells, in which RIFs were found to be associated with polymerized MreB (Oswald et al. 2016). Most importantly, RIFs could have crucial physiological roles, since the MreB-induced domains were shown to alter the localization of certain membrane proteins, and to affect the diffusion of lipid species and membrane proteins (Strahl et al. 2014; Oswald et al. 2016).

It, therefore, seems likely that differential fluidity of membrane regions, given by fatty acid composition, specialized lipid species and/or membrane-protein associations, organize physiological processes by controlling spatio-temporal localization of proteins. Moreover, this novel model of bacterial membrane organization begins
to resemble the eukaryotic membrane compartmentalization, where the cortical actin cytoskeleton organizes membrane compartments. This is achieved by creating a physical barrier for phospholipid and membrane protein diffusion (Kusumi et al. 2005; Andrade et al. 2015), and by affecting directly or indirectly the dynamics of lipid rafts (Viola and Gupta 2007). However, the bacterial MreB cytoskeleton is more permissive in terms of protein or lipid diffusion and organizes fluid membrane domains by a thus far unknown mechanism.

4.2 Lipid Rafts

In eukaryotic cells, signal transduction and membrane trafficking depend on the integrity of membrane assemblies that have differential lipid and protein composition and thereby physical properties, in comparison with the surrounding membrane (Simons and Toomre 2000; Pike 2003). These membrane domains, known as lipid rafts, are defined as sterol- and sphingolipid-enriched liquid-ordered lipid clusters with decreased fluidity. Sterols, such as cholesterol and ergosterol, favor a close packing of the acyl chains within the phospholipid bilayer, increasing membrane thickness and reducing its fluidity. In addition, the high affinity of cholesterol to the longer and saturated fatty acid chains of sphingolipids promotes their segregation from glycerophospholipids, forming more rigid structures in artificial bilayers that are suggested to resemble lipid rafts. Thus, the presence of sterols allows the phase separation within liquid-ordered domains, which have lesser fluidity. Also, an invariant component of lipid rafts is a group of proteins characterized by the stomatin, prohibitin, flotillin and HflK/C (SPFH) domain (Browman et al. 2007). SPFH-containing proteins are thought to be involved in protein recruitment to the rafts and in the anchoring of lipid rafts to the cortical actin cytoskeleton (Langhorst et al. 2005, 2007; Browman et al. 2007). Since SPFH-containing proteins are essential for eukaryotic lipid raft formation and they are found exclusively in these domains, they are commonly used as lipid raft markers.

4.2.1 Lipid Raft-like Domains in Bacteria

The fact that SPFH-containing proteins are widely distributed in prokaryotes, being present in more than 90% of bacterial genomes (Hinderhofer et al. 2009), raises the question of whether these proteins are able to drive the formation of lipid raft-like domains in bacterial membranes. And then, if such membrane structures exist in bacteria, how do they achieve the local increase of stickiness and decreased fluidity, characteristic of lipid rafts, in the absence of sterols and sphingolipids? Indeed, lipid raft-like domains, coined functional membrane microdomains (FMMs), have been identified in the cytoplasmic membrane of B. subtilis (López and Kolter 2010). In this study, Lopez and Kolter demonstrated that two SPFH-containing proteins, FloT (YuaG) and FloA (YqfA), which localize in discrete foci along the cell membrane, are spatially and functionally associated with a signaling pathway, which is involved in regulation of biofilm formation. Interestingly, this association was shown to depend on the activity of YisP, which catalyzes the formation of farnesol from
farnesyl diphosphate (Feng et al. 2014), suggesting that farnesol or farnesol-derived polyisoprenoid lipids might be needed for lipid raft-like domain formation in *B. subtilis*. Thus, the participation of specialized lipids that mimic the properties of sterols and sphingolipids and/or the involvement of proteins that affect the local fluidity of membranes may provide an answer to the above questions.

The tight packing of lipids and the phase separation characteristic of lipid rafts, render them resistant to the solubilization by nonionic detergents at low temperatures. Therefore, the biochemical characterization of lipid rafts has been largely pursued by the analysis of detergent-resistant membranes (DRM) in both eukaryotic and prokaryotic organisms. In *B. subtilis*, analysis of the DRM protein content revealed the presence of signaling proteins (histidine kinases and chemoreceptors), proteins of the cell wall biosynthesis machinery, transporters, and secretion proteins, along with SFPH-containing proteins (López and Kolter 2010; Bach and Bramkamp 2013). Some of these proteins have been shown to colocalize and directly interact with FloT and/or FloA. Moreover, functional dependence on flotillins was found for the histidine kinase KinC and for the Sec protein translocation apparatus (López and Kolter 2010; Bach and Bramkamp 2013; Schneider et al. 2015). Thus, similarly to lipid rafts in eukaryotic cells, bacterial FMMs appear to be involved in signaling and protein secretion.

SFPH containing proteins, or flotillins, seem to organize in whole the formation of bacterial FMMs. Indeed, flotillins were found to act as the scaffold for proteins that find the appropriate environment in FMMs to carry out kinetically favorable interactions and regulate their activities. In addition, analysis of Laurdan staining of *B. subtilis* cells showed that FloT and FloA form dynamic assemblies that can locally influence membrane fluidity, allowing liquid-ordered phase formation (Bach and Bramkamp 2013). This change in local fluidity was found to be dependent of YisP, indicating that polyisoprenoid lipids are required.

This body of recent data about FMMs in the model bacterium *B. subtilis*, advocates for the occurrence of bacterial lipid raft-like domains that are involved in many membrane-associated cellular processes. In analogy with eukaryotic lipid rafts, specialized lipid species, such as farnesol or its derived polyisoprenoid lipids, appear to be essential for FMM formation. By various approaches and to different extents of detail, lipid raft-like domains have been identified in other bacteria, such as *E. coli* (López and Kolter 2010; Guzmán-Flores et al. 2017), *Staphylococcus aureus* (López and Kolter 2010), *B. anthracis* (Somani et al. 2016), *Bacteroides fragilis* (An et al. 2011), *Borrelia burgdorferi* (LaRocca et al. 2013), and *Helicobacter pylori* (Hutton et al. 2017). *B. burgdorferi* and *H. pylori* are two of the few bacteria which possess cholesterol as a membrane component, despite that they do not carry cholesterol biosynthetic genes and therefore cannot carry out de novo sterol biosynthesis. Instead, both bacteria are able to obtain cholesterol from the host epithelial cells to generate glyco-cholesterol derivatives, which are incorporated into bacterial membranes. Interestingly, both bacterial species seem to form flotillin-dependent cholesterol-containing lipid raft microdomains that are assembled into the outer membrane (LaRocca et al. 2013; Hutton et al. 2017). *Bacteroides* species, which are common residents of the mammalian gut, form part of a small group of bacteria that possess
sphingolipids in their outer membranes. Remarkably, they can also use the host-provided cholesterol to form sphingolipid- and cholesterol-containing membrane domains, which have been shown to control stress response pathways. On the other hand, in Methylobacterium extorquens it was observed that, comparable to cholesterol in lipid rafts, hopanoids, lipid molecules present in many bacterial species, favor high order in the outer membrane.

The case for the model bacterium E. coli may be quite different. The E. coli genome encodes for 4 SPFH-containing proteins, which are all inner membrane integral proteins. These proteins are distributed in foci in different cell locations and are enriched in DRM fractions obtained from the inner membrane (López and Kolter 2010; Guzmán-Flores et al. 2017). Until now, E. coli is the unique Gram-negative bacterium in which inner membrane located lipid raft-like domains have been identified. Furthermore, E. coli lacks sterol, carotenoid and hopanoid lipids. Interestingly, the genome of some pathogenic E. coli strains, such as the enterotoxigenic E. coli B7A, carry a gene codifying for a putative serine palmitoyltransferase (Spt). Spt is responsible for the first step of sphingolipid biosynthesis in both bacteria and eukaryotic cells, suggesting that sphingolipids might exist in the membranes of these bacterial strains (Geiger et al. 2010). However, a functional or structural connection between sphingolipids and membrane domains in the above E. coli strains has not been reported thus far. Therefore, further work will be needed to determine the lipid composition of lipid raft-like domains and their protein cargo.

5 Conclusions and Research Needs

Lateral heterogeneity clearly occurs in bacterial membranes and it is essential for the coordination of a multitude of membrane-associated processes that occur simultaneously in a bacterial cell. Such cellular processes include signal transduction, transport, energy metabolism, cell division, cell wall metabolism, among others. The first notion of membrane domains in bacteria came from the observation of membrane regions enriched in anionic lipids, such as CL or PG. The occurrence and physiological importance of these domains has been largely discussed, and the irregular distribution of lipid dyes had diverse interpretations. However, membrane zones enriched in lipids with a determined head group could be related to the more recent view in which membrane heterogeneity is determined by differences in fluidity rather than by individual lipid species. In this respect, two different membrane domains have been described: membrane regions with increased fluidity and lipid raft-like domains, characterized by being less fluid (Fig. 3). Both types of domains coincide in bacterial membranes and seem to be organized by specific proteins, namely, the actin homologues MreB and the SPFH-containing proteins. In an early study, it was suggested that two types of membrane domains coexist in plasma membranes of E. coli and B. subtilis, one mainly lipid enriched and the other being a proteolipid domain (Vanounou et al. 2002). Interestingly, the authors interpreted their observations in a predictive manner, since it was not until 2010 that the coexistence of two types of membrane domains was demonstrated.
However, both types of membrane domains have important protein components and may require a complex lipid composition. This is exemplified by the fact that increase of local membrane fluidity has been proposed to require MreB in association with the cell wall precursor lipid II, whereas lipid raft segregation needs a liquid-ordered phase formation, which has only been observed in the presence of sterols, carotenoids, polyisoprenoids or hopanoids. Because these lipid species are absent in many bacteria further investigation is needed to determine the essential lipid components in lipid raft-like domains of these bacteria. In fact, CL has been shown to be associated to FFM, since CL enriched regions often colocalize with flotillin foci and CL molecules have been shown to copurify with this lipid raft markers (Donovan and Bramkamp 2009). However, CL depletion does not appear to affect FMMs formation, in contrast to eukaryotic cells and B. burgdorferi where no lipid rafts were formed after cholesterol depletion.

Efforts for the identification of specialized lipid species essential for membrane rafting in E. coli and other γ-proteobacteria are of uppermost importance. Taken into account that the integrity of FMMs has been shown to be essential for proper signal transduction by two component systems (TCSs) (Lopez and Kolter 2010, and own unpublished observations), and that many TCSs usually regulate the expression of virulence factors, the possibility to impede FMMs formation, e.g., by blocking a lipid biosynthetic pathway, becomes of special interest.

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